

1/2
AI
claims priority under 35 U.S.C. § 120 to, Application Serial No. 08/468,011, filed June 6, 1995 (now U.S. Patent No. 6,030,804 issued February 29, 2000). Each of the above-referenced applications is hereby incorporated by reference in its entirety. --

At page 6, line 9, immediately before the paragraph which begins "The following drawings..." please insert the following subheading:

-- Brief Description of the Drawings --

At page 6, please replace the paragraph beginning at line 12 with the following paragraph:

AI
-- Figure 1 shows the cDNA sequence (SEQ ID NO:1) and the corresponding deduced amino acid sequence (SEQ ID NO:2) of the G-protein PTH receptor of the present invention. The standard one-letter abbreviation for amino acids is used. Sequencing was performed using a 373 Automated DNA sequencer (Applied Biosystems, Inc.).--

At page 7, please replace the paragraph beginning at line 3 with the following:

AI
-- Figure 3 illustrates an amino acid alignment of the G-protein PTH receptor of the present invention (top line) and the human PTH receptor (bottom line). In Figure 3, the "Query:" line refers to the polypeptide sequence portion of the polypeptide according to the invention and the "Sbjct." line refers to the comparative portions from human PTH receptor protein. Further, in Figure 3 the polypeptide segments set forth in Query:729-908/Sbjct.:253-312; Query:909-1088/Sbjct.:313-372; Query:1089-1244/Sbjct.:373-424; Query:267-446/Sbjct.:102-161; Query:447-476/Sbjct.:162-171; Query:498-677/Sbjct.:177-236; Query:678-740/Sbjct.:237-257; Query:1248-1424/Sbjct.:427-485; Query:159-269/Sbjct.:24-60; and Query:1508-1576/Sbjct.:512-534 correspond to SEQ ID NOS:9-28, respectively.--

At page 7, please replace the paragraph beginning at line 7 with the following:

As -- In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 97186 on June 1, 1995 with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, VA 20110-2209 under terms of the Budapest Treaty. --

At page 34, please replace the paragraph beginning at line 32 and continuing to page 36, with the following:

As -- The DNA sequence encoding for PTH receptor, ATCC No. 97186 is initially amplified using PCR oligonucleotide primers corresponding to the 5' and sequences of the processed protein and the vector sequences 3' to the PTH receptor gene. Additional nucleotides corresponding to the PTH receptor were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence CAGCCGTCGCCGGGCTTGGCCTGG (SEQ ID NO:3) contains a SmaI restriction enzyme site followed by 6 nucleotides of the PTH receptor coding sequence starting from the presumed second amino acid of the processed protein codon. The 3' sequence CCTCAGTGTCGACTTGTCATCCTTCAG (SEQ ID NO:4) contains complementary sequences to Sal I site and is followed by 6 nucleotides encoding the PTH receptor. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector PQE-30. (Qiagen, Inc. Chatsworth, CA, 91311). PQE-30 encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. PQE-30 was then digested with SmaI and Sal I. The amplified sequences were ligated into PQE-30 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli available from Qiagen under the trademark M15/rep 4 by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones

1
Orig
A5

containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.⁶⁰⁰) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalactopyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized PTH receptor was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). The PTH receptor was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate. --

At page 37, please replace the paragraph beginning at line 3 with the following:

AB

-- The DNA sequence encoding for PTH receptor, ATCC No. 97186, was constructed by PCR using two primers: the 5' primer (GTTGGCATATTGGAAGCTTTTTCGGG) (SEQ ID NO:5) contains a Hind III site 5 UTR; the 3' sequence (CAGTTTCTAGATGTCATCCTTCAGTGTC (SEQ ID NO:6) contains complementary sequences to XbaI site, translation stop codon, and the last 12 nucleotides of the PTH receptor coding sequence (not including the stop codon). Therefore, the PCR product contains a Hind III site, PTH receptor coding sequence followed by a translation termination stop codon and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNA3/Amp, were digested with Hind III and XbaI restriction enzyme and ligated. The ligation mixture was transformed into E. coli strain DH5 α (available from Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant PTH receptor,

Cold
A6
COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the PTH receptor HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with ³⁵S-cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media were precipitated with a HA specific monoclonal antibody. Proteins preecipitated were analyzed on 15% SDS-PAGE gels. --

At page 38, please replace the paragraph beginning at line 4 with the following:

-- The DNA sequence encoding the full length PTH receptor protein, ATCC No. 97186, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene: --

At page 38, please replace the paragraph beginning at line 8 with the following:

-- The 5' primer has the sequence TCCTACCCGGGCGCCATCATGGC CTGGCTGGGGGCCT (SEQ ID NO:7) and contains a SmaI restriction enzyme site (in bold) followed by 8 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (J. Mol. Biol. 1987, 196, 947-950, Kozak, M.), and just behind the first 19 nucleotides of the PTH receptor gene (the initiation codon for translation "ATG" is underlined).--

At page 38, please replace the paragraph beginning at line 16 with the following:

-- The 3' primer has the sequence CAGTTTCTAGATGTCATCCTTCAGTGTC (SEQ ID NO:8) and contains the cleavage site for the restriction endonuclease XbaI and 13 nucleotides complementary to the 3' non-translated sequence of the PTH receptor gene. The amplified sequences were isolated from a 1% agarose gel using a commercially

cont
A9

available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases SmaI and XbaI and then purified. This fragment is designated F2.

At page 38, please replace the paragraph beginning at line 25 with the following:

A10

-- The vector pRG1 (modification of pVL941 vector, discussed below) is used for the expression of the PTH receptor protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases SmaI and XbaI. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).--

At page 39, please replace the paragraph beginning at line 11 with the following:

A11

-- The plasmid was digested with the restriction enzymes SmaI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel as described in Example 1. This vector DNA is designated V2.--